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Transatlantic disjunction in fleshy fungi III: *Gymnopus confluens*

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Abstract

Phylogeographic data indicate that DNA differences consistently exist between the North American and European allopatric populations of *Gymnopus confluens*. Conversely, pairing experiments show that collections from both populations were sexually compatible *in vitro* and detailed morphological examinations of numerous fresh and dried basidiomata do not produce qualitative differences. Percent ITS sequence divergence between Europe and North American collections of *G. confluens* was 3.25%. Species delineation metrics including Rosenberg's P_{AB} statistic, P_{ID} metrics, R_{RD} (randomly distributed) and PTP (Poisson Tree Processes) gave mixed indications that North American and European populations were distinct at species rank. The North American populations are described as *Gymnopus confluens* subsp. *campanulatus* (Peck) R.H. Petersen.

Key words

Allopatric speciation, biogeography, disjunct distributions, phylogenetic species, species delineations

Introduction

North American species numbers for fleshy fungi are certainly underestimated by the historical practice of assigning European names to North American taxa but the extent to which North American taxon numbers are underestimated is unknown and is dependent on species concepts used by investigators. A number of studies documenting cryptic geographical species argue that species may be more localized and more numerous than historical treatises might suggest. A recent study by Talbot et al (Talbot et al. 2014) demonstrated that most soil fungi are regionally endemic and limited by

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factors such as climate and dispersal ability. Geographical partitioning may be the rule for basidiomycete taxa, rather than the exception (Taylor 2008; Taylor et al. 2000).

In agaric systematics, discrepancy among parameters used to make taxonomic judgments at species rank is becoming more widely recognized. Three such standards, DNA sequence data, sexual compatibility and morphological characters of basidiocarps have evolved as important in taxonomic judgments, including proposal of new taxa [*Lentinus* (Grand et al. 2011); *Megacollybia* (Hughes et al. 2007); *Lentinellus* (Hughes and Petersen 2004; Petersen and Hughes 2004); *Sparassis crispa* complex (Hughes et al. 2014); *Artomyces* (Lickey et al. 2003)], and revision of generic or species complexes [(*Panellus stypticus* (Jin 2001); *Marasmius scorodonius* (Gordon and Petersen 1998); *M. androsaceus* (Gordon and Petersen 1997); *Strobilomyces* (Sato et al. 2007); *Omphalotus* (Kirchmair et al. 2004; Petersen and Hughes 1998); *Pleurotus* (Vilgalys et al. 1993; Vilgalys and Sun 1994); *Gymnopus* s. l. (Mata et al. 2007)]. As phylogenetic analyses based on molecular data have increased, it has become increasingly clear that genetic differentiation of fungi may proceed in the absence of observable morphological change in basidiocarps. Taylor et al. (2006) note that eukaryotic microbes (predominantly fungi) have few discriminating morphological characters on which to base species assessments. Further, the basidiomycete fungal mating system acts to slow or prevent establishment of reproductive barriers (James et al. 1999). In the absence of morphological change and reproductive isolation, delineation of taxa may rest on evaluation of observed genetic change among populations.

Few primary literature sources are available for identification of *Gymnopus s.str*. Peck's proposals of new species, often under *Marasmius*, were included by Halling (1983) in a publication intended to summarize the taxa (under *Collybia*) of northeastern USA and adjacent Canada. Murrill (Kimbrough 1972) accepted *Gymnopus* Roussel to represent the "collybioid" taxa of Florida and described several taxa of *Gymnopus* (i.e. *Collybia*) and *Marasmius* s.l. from central Florida [see Kimbrough (1972); Halling (1983)]. This left a geographical hiatus between the New England region and Florida. Without publishing a summary of his study, Hesler included observations on Murrill specimens in his (Hesler) notebooks (http://trace.tennessee.edu/utk_hesler/), and Smith [see (Petersen et al. 2014)] also examined Murrill's type specimens. Lennox (1979) published her dissertation on Pacific Northwest collybioid genera, but included only a single species under *Collybia* s. str. Desjardin (1987) published a summary of collybioid fungi from California which further expanded geographical and taxonomic coverage of Gymnopus.

In Europe, several compendia were available (Kühner and Romagnesi 1953; Moser 1978); but Antonín and Noordeloos (Antonín and Noordeloos 1993; Antonín and Noordeloos 2010) and Antonín et al. (2013) summarized *Gymnopus* for Europe and arranged the taxa according to Mata et al.'s recent phylogenetic treatment (Mata et al. 2007).

A molecular discontinuity between European and eastern North American populations of *Gymnopus confluens* has been known for some years (Mata et al. 2007), most recently implied in a phylogeny placing *G. eneficola* from Newfoundland, Canada (Petersen et al. 2014). Additional data now permit a more detailed report as part of a wider project on transatlantic disjunctions in fleshy fungi.

In this study, we evaluate morphology, ability to dikaryotize *in vitro* and ITS-LSU sequence divergence to determine whether intercontinental allopatric populations of *Gymnopus confluens* represent separate taxa at some rank. In this paper, we consider the term population to comprise a group of currently interbreeding individuals. This paper is third in a series of papers exploring the nature of transatlantic disjunctions. The first in the series was Hughes et al. (2014), the second is Petersen et al. (in press).

Methods

Collections

Field collections made by the authors were dried overnight on the day they were collected. Prior to drying, a fragment was stored in silica gel for later DNA extraction and spores were deposited on malt extract agar (MEA) plates to obtain cultures. Collections were accessioned into TENN, cultures into CULTENN.

Pairing experiments

Establishment of single basidiospore isolates (SBIs) and pairing experiments were performed as described in Gordon and Petersen (1992). All SBIs were examined microscopically to determine monokaryon status before mating experiments.

Macromorphology

Macromorphological characters observed were stipe length, stipe vesture, color of living and dried material, and lamella structure (distance between lamella, number, narrow vs. broad, attachment). "Complete" lamellae (those which extended from pileus margin to attachment juxtaposed to stipe) were interspersed with numerous lamellulae, usually of at least two and occasionally three ranks. The most accurate assay counted all lamellae (and lamellulae) which reached the pileus margin. This tally was performed by counting lamellae for approximately ¼ pileus circumference and multiplying by four.

Micromorphology

Basidiospore statistics were gathered for "European" vs. "American" collections. Two spore metrics were especially examined: Q^m (median ratio of spore length to width) and L^m (median spore length). Pileipellis hyphae were examined for presence of short side branches reported by Antonín and Noordeloos (2010). Terminal cell shape of

these side branches was noted. Cheilocystidia vary within subg. *Vestipedes*, and were evaluated for *G. confluens* in both size and shape (i.e.non-strangulate to strangulate).

Molecular studies

DNA was extracted from dried specimens and/or cultures, and ITS and LSU sequences were amplified and sequenced as described in Hughes et al. (2013). Sequences were deposited in GenBank (Table 1). Sequences were manually aligned using GCG (2000). PhyML with 100 bootstrap replicates was performed in Geneious V9. *Gymnopus eneficola* (Petersen et al. 2014) was selected as the outgroup because it is the most closely related species and is sister to *G. confluens*. *G. eneficola* is often mistaken for *G. confluens* in the field. Trees were visualized in TreeView (Page 1996) and deposited in Dryad (Petersen and Hughes 2015).

Species-delineation metrics

Several species-delineation metrics including Rosenberg's P_{AB} statistic (Rosenberg 2007), P_{ID} (strict) and P_{ID}(liberal) (Ross et al. 2008), P_{RD} (Rodrigo et al. 2008) and PTP (Zhang et al. 2013). P_{AB}, P_{ID} (strict), P_{ID} (liberal) and P_{RD} were implemented in Geneious V9 (Geneious 2005; Masters et al. 2011). PTP was implemented at http://species.h-its.org/ using web default settings for generations (100,000) burn-in (0.1, MCMC convergence was reached) and thinning (100). Rosenberg's P_{AB} statistic is the probability that a putative species will be monophyletic with respect to a sister clade under the model of random coalescence. The null hypothesis is that monophyly is a chance outcome of random branching. The P_{ID} statistics provide the frequency with which a member of a putative species can be correctly identified given a specific alignment of sequences. P_{ID}(strict) requires that an unknown specimen falls within but not sister to the species clade. P_{ID}(liberal) requires that an unknown specimen falls either sister to or within the species clade. P_{RD} (Probability Randomly Distributed) is the probability that a clade has the observed degree of distinctiveness due to random coalescent processes. A probability value less than 0.05 rejects the null hypothesis of random coalescence and suggests that the clade is a cryptic species. PTP (Poisson Tree Processes; Zhang et al. 2013) estimates the number of species using both maximum likelihood and Bayesian approaches.

Specimens examined for morphological analysis

NORTH AMERICA, **CANADA**, **New Brunswick**, Fundy National Park, vic. Alma, Caribou Plains Trail, 45°38.59'N, 65°06.94'W, 25.IX.2013, coll Stephen Clayden, TFB14409 (TENN-F-69073); Fundy National Park, vic. Alma, Maple Grove Backroad, 45°35.34'N, 64°59.014'W (stop 1), 24.IX.2013, coll. Unknown, TFB 14389

Table 1. Sequences used in phylogenetic reconstructions.

Herbarium No.	Collection No.	GenBank ITS	GenBank LSU	Location1
		Gymnopus eneficola		
MICH PK6975 (as *G. confluens*)	PK6975	KP710270	KP710304	USA, AK
MICH PK6976 (as *G. confluens*)	PK6976	KP710271	KP710305	USA, AK
No specimen	MS4-007	KJ416257	No sequence	Canada, NL
TENN-F-69120	MR3-016	KJ128262	No sequence	Canada, NL
TENN-F-69122	10-09-21 AV04	KJ128265	KJ189590	Canada, NL
TENN-F-69123	09-09-26 AV13	KJ128264	KJ189586	Canada, NL
TENN-F-69127	06-09-02 AV01	KJ128267	KJ189588	Canada, NL
TENN-F-69128	09-09-26 AV12	KJ128268	KJ189589	Canada, NL
		Gymnopus confluens		
No specimen	House dust[c]	AM901885	No sequence	Finland
No Specimen	MS4-009	KP710277	No sequence	Canada, NL
BRNM734005[d]	BRNM734005	JX536124	No sequence	Czech Rep.
Culture LE(BIN)	10977ss7[a] and LE(Bin)183	DQ450047	No sequence	Russia, Southern Urals
Culture LE(BIN)	LE(BIN)1178	KP710282	KJ189580	USA, NC
Culture LE(BIN)	LE(BIN)1212	KP710290	KJ189575	Russia, Leningrad area
Culture LE(BIN)	LE(BIN)2294	KP710291	KJ189576	Russia, Altai Region
Culture LE(BIN)	LE(BIN)2357	KP710287	KJ189577	Russia, Altai Region
EIU ASM10643	ASM10643	KP710303	No sequence	Russia, Samara Region
MICH PK6820	PK6820	KP710286	KP710311	USA, AK
MICH PK6943	PK6943	KP710285	KP710312	USA, AK
Not given	H21[b]	JX029935	No sequence	Czech Rep.
Private collection, Michael Burzynski	BUR1	KP710275	KJ189583	Canada, NL
TENN-F-50524	3787	DQ450044	No sequence	Sweden
TENN-F-52248	5824	DQ450053	No sequence	USA, WA
TENN-F-53522	7219	KP710283	KP710309	USA, NC
TENN-F-55695	9048ss2[a]	DQ450050	No Sequence	USA, CA
TENN-F-55879	6960ss3[a]	KP710302	No sequence	Scotland
TENN-F-55879	6960ss4[a]	KP710301	No sequence	Scotland
TENN-F-55880	6962	DQ450051	No Sequence	Scotland
TENN-F-58239	10650ss4[a]	DQ450046	No sequence	Russia, Leningrad
TENN-F-58242	10653	AY256697	No sequence	Russia, Leningrad
TENN-F-59219	11335ss2[a]	DQ450045	No sequence	France, Rhône-Alpes
TENN-F-59285	11400ss1[a]	KP710299	No sequence	Switzerland
TENN-F-59500	9875	AY505773	No sequence	USA, WA
TENN-F-59578	11615	DQ450048	No sequence	Russia, Novgorod
TENN-F-59582	11619	DQ450049	No sequence	Russia, Novgorod
TENN-F-59603	11641	KP710300	No sequence	Russia, Novgorod
TENN-F-60062	12134	DQ450052	No Sequence	USA, NC, GSMNP
TENN-F-60736	11852	KP710289	No sequence	Russia, Kedrovaya Res.

Herbarium No.	Collection No.	GenBank ITS	GenBank LSU	Location1
TENN-F-61147	12587	FJ596784	No Sequence	USA, NC, GSMNP
TENN-F-63806	PBM2991	KP710276	KP710310	USA, VA
TENN-F-65121	13744h1[a]	KP710297	KJ189572	Belgium
TENN-F-65121	13744h2[a]	KP710298	KJ189572	Belgium
TENN-F-65131	13754	KP710288	KJ189571	Belgium
TENN-F-65835	13939	KP710284	KJ189579	USA, NY
TENN-F-67819	14072	KP710280	KJ189258	USA, NC
TENN-F-67822	14075	KP710281	KJ189581	USA, NC
TENN-F-67864	14114h1[a]	KP710295	KJ189573	Germany, Thuringia
TENN-F-67864	14114h2[a]	KP710296	KJ189573	Germany, Thuringia
TENN-F-67865	14115	KP710292	KJ189578	Germany, Thuringia
TENN-F-67882	14132h1[a]	KP710293	KJ189574	Germany, Thuringia
TENN-F-67882	14132h2[a]	KP710294	KJ189574	German, Thuringia
TENN-F-69053	14389	KP710279	KJ189584	Canada, NB
TENN-F-69073	14409	KP710278	KJ189585	Canada, NB
WTU005	WTU005	KP710273	KP710307	USA, AK
WTU394	WTU394	KP710274	KP710308	USA, AK
WTU514	WTU514	KP710272	KP710306	USA, AK

[a]ss=single spore culture, available from the culture collection of the University of Tennessee (CulTENN); h1, h2=haplotypes deduced from forward and reverse sequences; [b] Baldrian et al. 2013; [c] Pitkaranta et al. 2007; [d] Antonín et al. 2013

(TENN-F-69053); **Newfoundland**, Moccasin Lake, Abitibi Trail, 10.ix.2008, coll. Maria Voitk, mixed woods, MS4-009 (TENN-F-69133). **UNITED STATES**, **New York**, Tompkins Co., Ringwood Preserve, 42°27.03'N, 76°21.80'W, 4.IX.2013, coll TJ Baroni & RHP, TFB 14357 (TENN-F-69006); Tompkins Co., vic. Dryden, Ringwood Preserve, 42°28.11"N, 76°19.06"W, 13.IX.1984, leg. & det. R.E. Halling, REH no. 3851 (dupl. NY), TENN-F-47030; **North Carolina**, Macon Co., vic. Highlands, Shortoff Mt. area, 35°05.47'N, 083°11.25'W, 18.VII.1994, coll. J. Johnson, TFB7219 (TENN-F- 53522)[annot. R.E.Halling as C. confluens]; **Tennessee**, Sevier Co., GSMNP, Rainbow Falls Parking Area, 28.VII.1989, coll RHP (as Collybia ?acervata), TFB 2033 (TENN-F-48376); Sevier Co., vic. Gatlinburg, GSMNP, "Mt. LeConte," 8.VIII.1941, coll L.R. Hesler & S.L. Meyer, LRH 13883 (TENN-F-13833); **Virginia**, Smyth Co., vic, Sugar Grove, Mt. Rogers National Recreation Area, Appalachian Trail, 8.VIII.2008, coll. P.B. Matheny, PBM 2991 (TENN-F-63806).

EUROPE, **BELGIUM**, Domain Massembre vic. Hevre, 50°09.62' N, 4°51.48'E, 7.IX.2010, coll RHP, TFB13754 (TENN-F-65131). **FINLAND**, Hämeenlinna, Torronguo National Park, 60°44.32'N, 23°38.63'E, 8.VIII.2002, coll. J.L. Mata, TFB 11055 (TENN-F-59469). **FRANCE**, Rhône-Alpes, Dpt. Savoie (73), commune St. Germain laChambotte, 45°46.62'N, 5°53.07'E, 10.IX.2001, RHP & Pierre-Arthur Moreau, TFB11335 (TENN-F-59219). **GERMANY**, Thuringia, vic. Menteroda, 51°18.04'N, 10°31.44'E, 28.VIII.12, coll RHP 2012, TFB14132 (TENN-F-67882); Thuringia, vic. Schlotheim by Pöthen, 51°16.90'N, 10°33.434' E, 27.VIII.2012, coll. RHP, TFB14115

(TENN-F-67865). **RUSSIA**, Leningrad Reg., vic. Lodeynoe Pole, 60°41.70'N, 33°17.98'E, 30.VIII.1999, coll RHP, TFB10650 (TENN-F-58239); Novgorod Region, Valdai District, National Park Valdaiski, vic. Road to National Park, 57°57.88'N, 33°19.32'E, 19.VIII.2003, coll. RHP, TFB 11615 (TENN 59578); Valdai National Park at resort, 58°00.511' N, 33°21.543' E, 22.VIII.2003, coll. RHP, TFB11641 (TENN-F-59603); Samara Region, Bakhilovo district, vic. Bakhilovo, Shiryayeva Valley, 53°24.30'N, 49°55.03'E, 17.VIII.2004, coll. RHP, TFB12171 (TENN-F-60109). **SWEDEN**, Uppland, vic. Uppsala, Gottsundaborgen, 59°48.8'N, 17°37.40'E, 7.IX.1994, coll Svenngunnar Ryman, TFB7262 (TENN-F-53546). **SWITZERLAND**, Graubunden, Chur, Lenzerheide, 46°40.17'N, 9°38.97'E, 19.IX.2001, coll. E. Horak, TFB11400 (TENN-F-59285).

Abbreviations used in table and figures

North America — AK=USA, Alaska, GSMNP=USA, Great Smoky Mountains National Park (TN or NC); NB=Canada, New Brunswick; NC=USA, North Carolina; NL=Canada, Newfoundland; TN=USA, Tennessee; VA=USA, Virginia; WA=Washington. **Europe** — BE=Belgium; FI=Finland; FR=France; GE=Germany; RU=Russia; SZ=Switzerland; SW=Sweden. SBI=single-basidiospore isolates; h=haplotype; c=clone.

Data resources

The data underpinning the analyses reported in this paper are deposited in the Dryad Data Repository at doi: 10.5061/dryad.8239h.

Results

Gymnopus confluens basidiomata from North America and Europe are shown in Figs 1, 2.

Morphological parameters – macromorphology

Macromorphological characters readily distinguish *G. confluens* (at least in Europe and North America) from other *Gymnopus* taxa. In nature and in herbarium specimens, basidiomata generally exhibit long stipes compared to pileus diameter.

Lamellae: Lamellae in *G. confluens* appear to be quite consistent; crowded, narrow and significantly seceding upon drying. In an attempt to statistically measure the first two items, lamellae in numerous collections were carefully examined for breadth (rarely exceeding two mm) and number. The number of lamellae reaching pileus margin ranged from 116–147, consistently more than 120, and with no discernible intercontinental difference. In similar morphological taxa (i.e. *G. subnudus*, *G. eneficola*, etc.) this number ranged from 65–87, significantly fewer than in *G. confluens*.

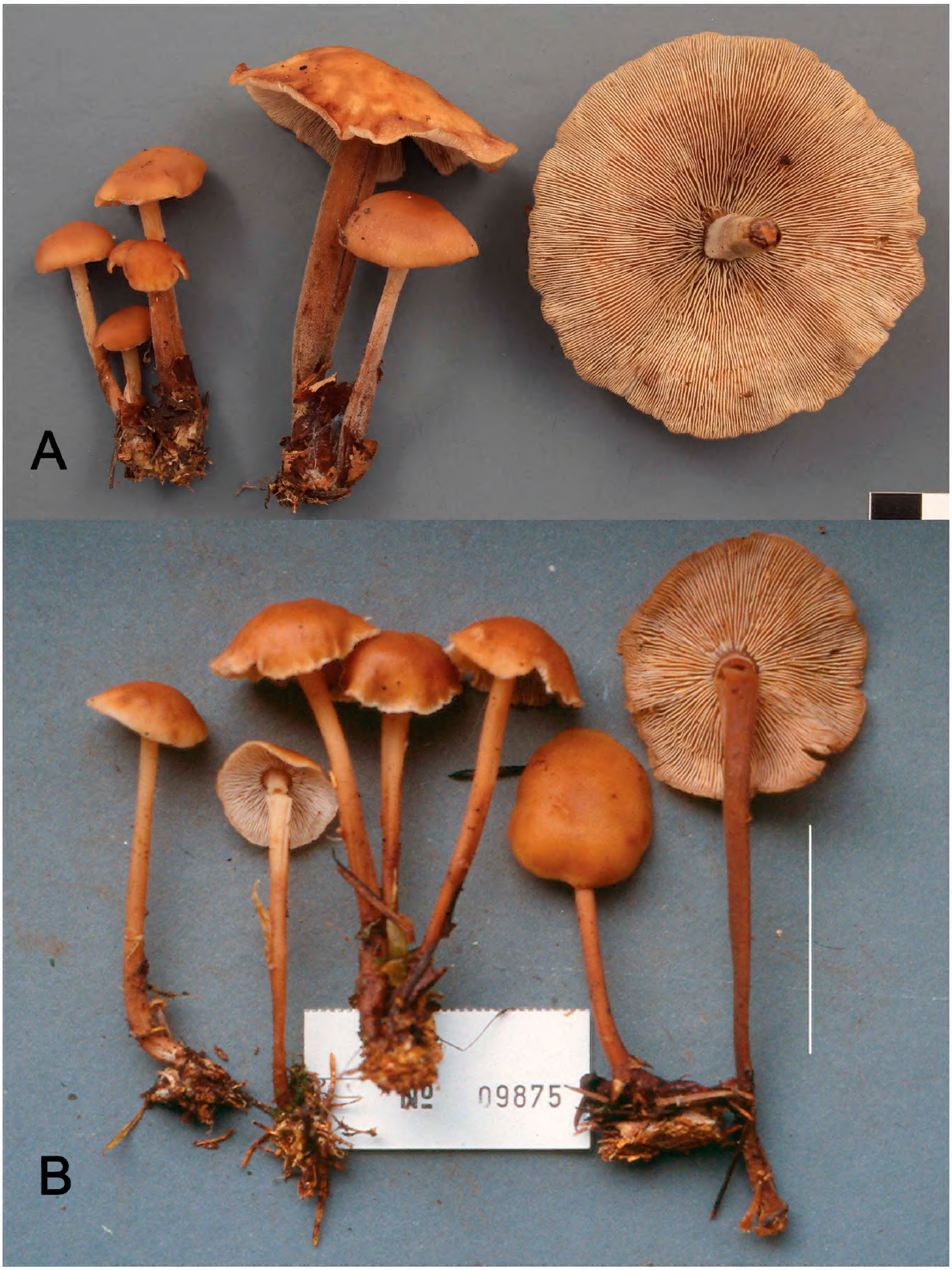

Figure 1. *Gymnopus confluens* subsp. *campanulate* basidiomata, North America. **A** TENN-F-69073 (TFB14409) NB. Photo courtesy Roger Smith. Black box = 10 mm **B** TENN-F-59500 WA. Standard bar = 50 mm. Basidiomata in both images exhibit a tendency toward campanulate pileus margin.

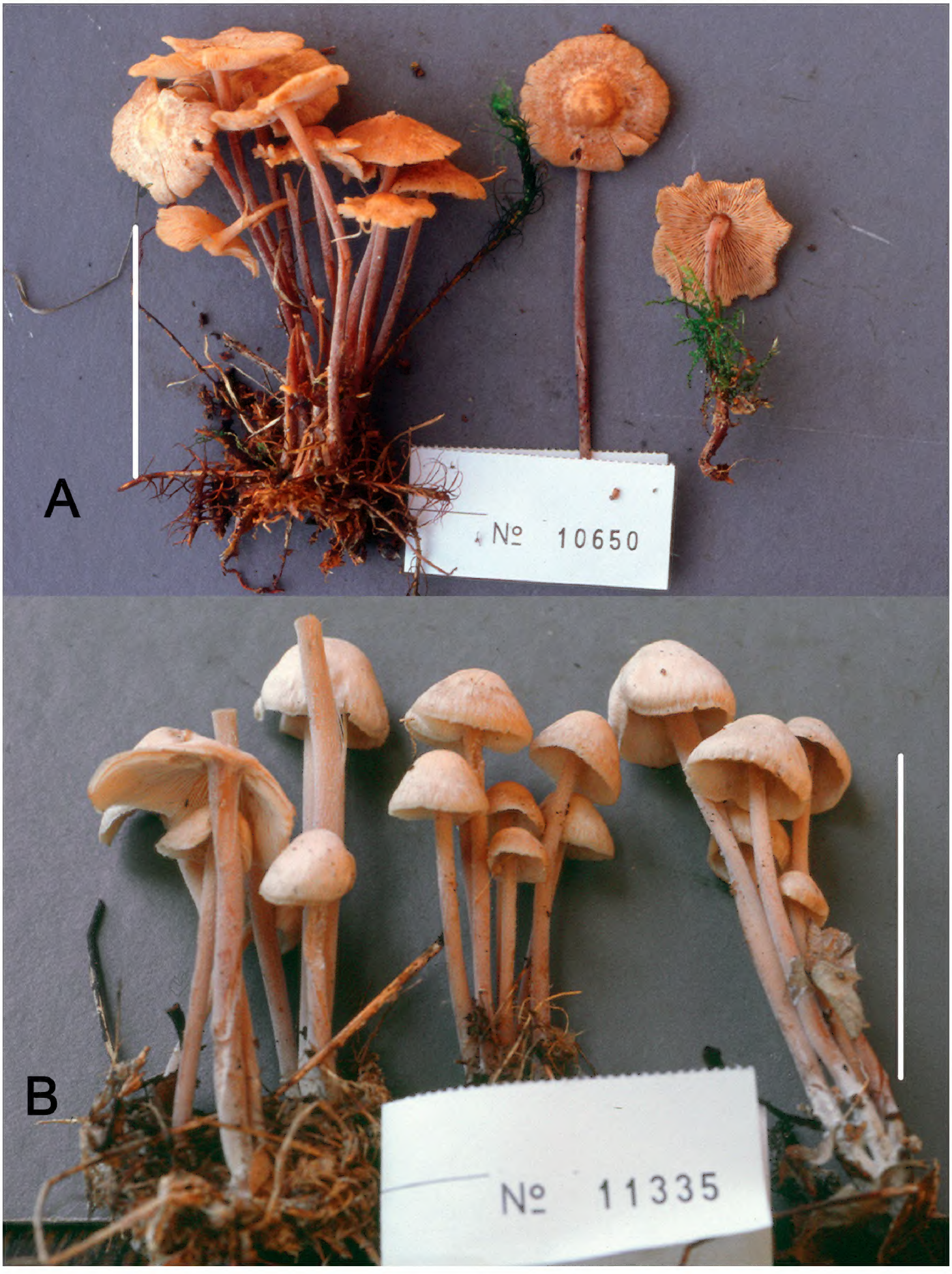

Figure 2. *Gymnopus confluens* basidiomata, Europe. **A** TENN-F-58239 (TFB10650) RU **B** TENN-F-59219 (TFB11335) FR. Standard bars = 50 mm.

Stipe: Pileus Ratio: Overall, stipe length:pileus diameter usually exceeded 3:1 (with range from 1.5:1 to 6:1). This ratio varied little between North American and European populations, but both clades exhibited some ratios downward, usually explainable due to dry weather or poor nutrition.

Stipe vesture: In most cases, stipe vesture is most sparse upward on the stipe and there (at 10X) exhibiting densely distributed spikes. Downward, vesture becomes denser, and toward the stipe base, a felty subiculum subsumes individual spikes and often is strigose. In dried material, stipe vesture takes on a gray coloration, sometimes with a very slight olive tint. Stipe vesture varied considerably in both populations/clades. In fact, vesture variation within the major populations exceeded that between clades. No suitable metric was devised to summarize this situation, but macroscopic vesture characters did not prove distinctive.

Morphological parameters – micromorphology

Variation in spore shape is shown in Figs 3 and 4. Spores of at least three European collections were shorter (and somewhat narrower) than those of eastern North American material, and also tapering more obviously proximally (TENN-F-67865 GE, TENN-F-67882 GE, TENN-F-59212 FR). Spores of most European collections approach the metrics of eastern North American material (Table 2). A few collections of otherwise mature basidiomata were devoid of spores. Whether this is a function of *in vivo* drying followed by mechanical but not biological resuscitation is not known. Counter to this hypothesis is the presence on such basidiomata of mature (sterigmate), turgid basidia which appear to be fecund.

When spore statistics from numerous collections were compared, little difference was apparent, and spore statistics were concluded to be inconclusive for morphological separation of the phylogenetic clades of *G. confluens*. Median spore length for North American collections was 7.72 µm (n=10 collections); for European collections it was 7.52 µm (n=10 collections) (Table 2). This was not significantly different based on a 2-tailed T-test (P=0.40, df=19). Q^m for North American collections was 2.34 (n=10 collections); for European collections it was 2.30µ (n=14 collections). Q^m values were not significantly different based on a two-tailed T-test (P=0.71). The two collections from Alaska (MICH139598 and MICH139602) which clearly fell within the European clade by ITS sequence, were included in calculations of spore statistics for Europe.

Cheilocystidia: Cheilocystidum size and shape vary within *Gymnopus* subg. *Vestipedes*, and this variation was closely examined for numerous collections of *G. confluens* from both continents. Variation in both size and shape (i.e.non-strangulate to strangulate) was expected based on previous reports and illustrations. As expected, cheilocystidia varied in abundance, size and shape, but without correlation to geographic origin. Although cheilocystidia of European collections generally appear to be longer and longer-stalked than those from eastern North American specimens, the range of sizes and complexities seems parallel across the two populations. Overall variation of cheilo-

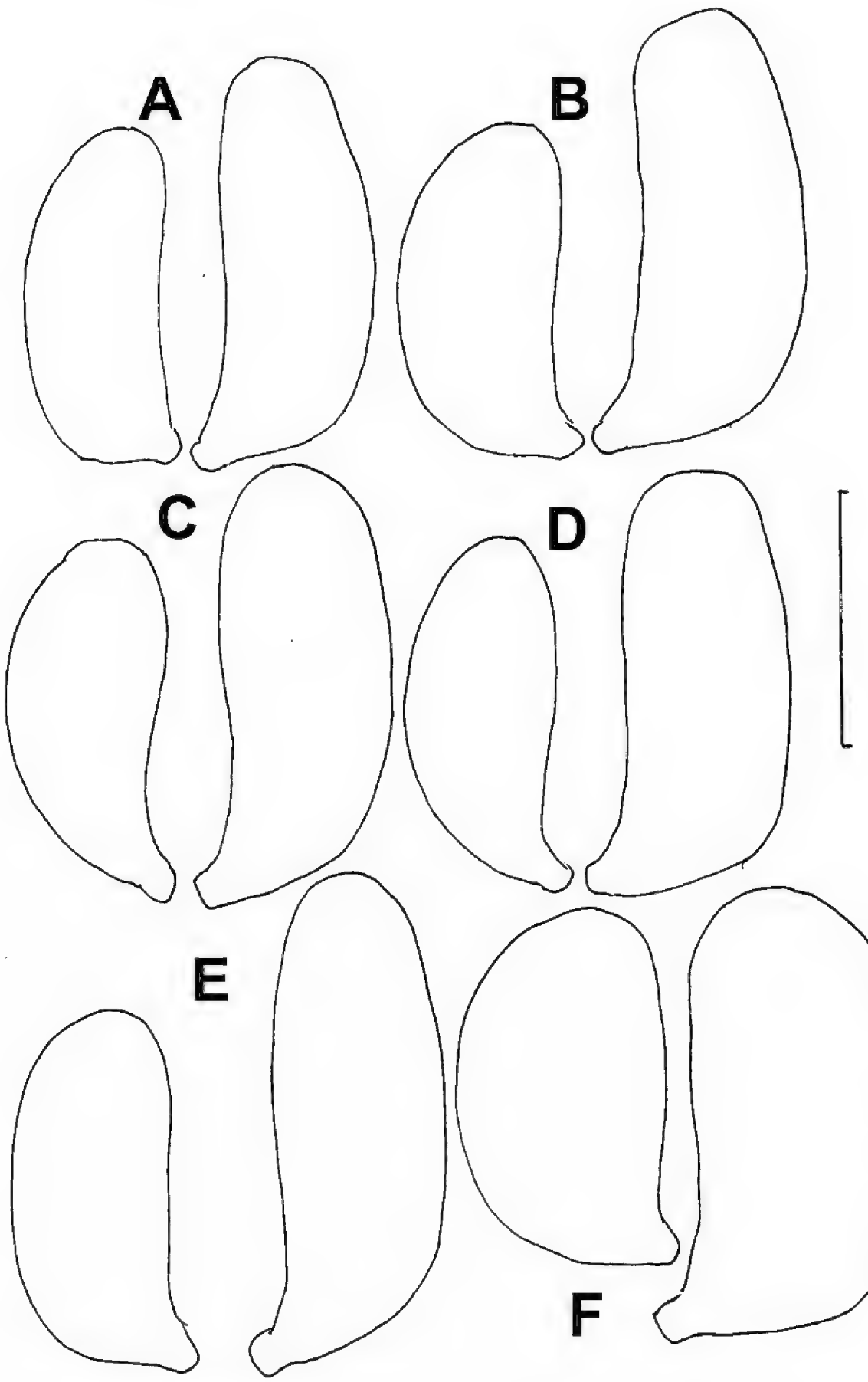

Figure 3. *Gymnopus confluens* Europe. Outlines of basidiospores showing variation in sizes and shapes. **A** TENN-F-59219 FR **B** TENN-F-67865 GE **C** TENN-F-65131 BE **D** TENN-F-59578 RU **E** TENN-F-53546 SW **F** TENN-F-59285 SZ. Standard bar = 5 µm.

cystidia is shown in Figs 5 and 6, where cheilocystidia are arranged according to increasing complexity. Tentacularly branched cheilocystidia (TENN-F-53546 SW) and the formation of a thatch out of the withered cheilocystidial apices (TENN-F-60109 RU) seem unique in *Gymnopus* sect. *Vestipedes*. Cheilocystidia may be involved in mi-

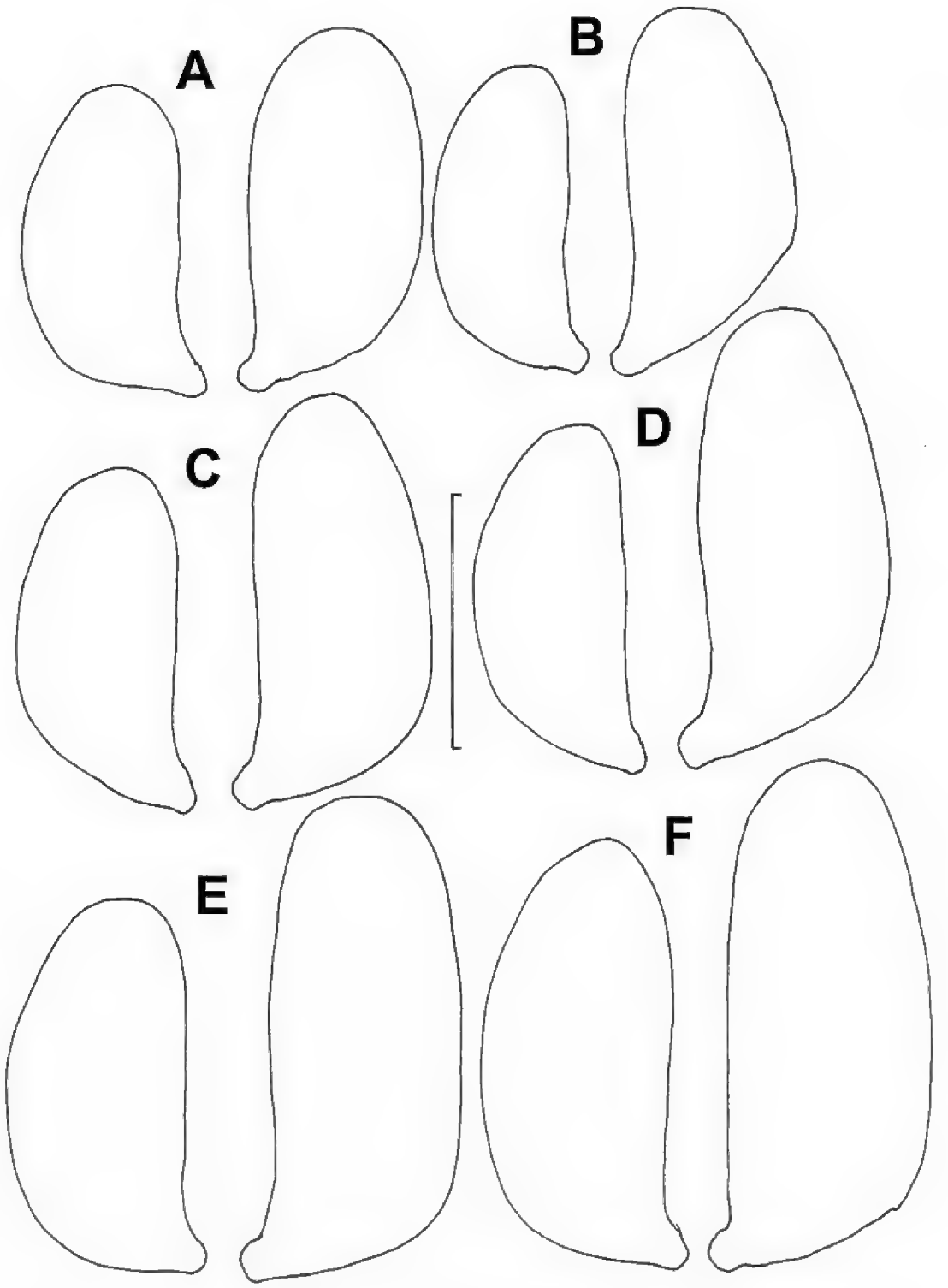

Figure 4. *Gymnopus confluens* subsp. *campanulate*. North America. Outlines of basidiospores showing variation in sizes and shapes: **A** TENN-F-47030 NY **B** TENN-F-48376 TN **C** TENN-F-63806 VA **D** TENN-F-48376 TN **E** WTU-F-021394 AK **F** WTU-F-024005 AK. Standard bar = 5 µm.

croscopic exudate of slime which conserves water as a microscopic moist chamber in which the apical portion of cheilocystidia can proliferate.

Caulocystidia: In observing caulocystidial hyphae composing stipe vesture, especially those gathered to form the characteristic vesture "spikes," some variation was perceived in the shape of the terminal cells, whether equal (parallel-sided and bluntly rounded at apex), tapering distally (and therefore narrowly rounded at apex), or some variation in shape (i.e. subsagitate, lobed, etc.). Once noted, special care was taken to observe this character. Caulocystidial hyphae are invariably clamped within their emergent length. Caulocystidia, correctly depicted by Antonín and Noordeloos (2010)

Table 2. Basidiospore metrics for European and North American collections of *Gymnopus confluens*.

Collection Accession Number[4]	Spore Length × width (µm)	L^m (µm) (median length)	Q (ratio length to width)	Q^m (median Q values)
TENN-F-50359 SZ[1]	6.5–8.5(–9) × (2.5–)3–3.5	7.30	1.86–3.00	2.35
TENN-F-53546 SW[1]	(6–)7–8(–8.5) × (3.5–)3.5–4.5	7.40	1.71–2.67	2.02
TENN-F-53546 SW[2]	7.5–9.5(–10) × 3.5–4	8.55	2.13–2.71	2.31
TENN-F-59219 FR	(5.5–)6–7(–8) × (2.5–)3–3.5	6.50	2.00–2.33	2.13
TENN-F-59282 SZ[1]	7.5–8.5 × 3.5–4	7.90	1.88–2.14	2.06
TENN-F-59282 SZ[2]	(7.5–)8.5–10 × 3.5–4	8.70	2.00–2.71	2.32
TENN-F-59578 RU	7–9 × 3–4	8.00	2.13–2.83	2.37
TENN-F-65131 BL	(6–) 6.5–8 × 3–3.5(–4)	7.15	1.71–2.50	2.11
TENN-F-67865 GE	(5.5–)6–7 × 2.5–3.5	6.40	1.86–2.80	2.32
TENN-F-67882 GE	6–7 × 3–3.5	6.40	1.71–2.00	1.94
TENN-F-62904 SW	(5.5–)7.5–9.5 × 2.5–3.5(–4)	7.85	2.00–3.20	2.61
TENN-F- 50565 SW	7.5–9 × 2.5–3.5	8.20	2.33–3.60	2.85
MICH 139598 AK[3]	6.5–8.5 × 3–4	7.45	1.88–2.50	2.17
MICH 139602 AK[3]	7.5–9(9.5) × 3–3.5(4)	8.25	2.13–3.17	2.60
		$L^{m\ ave}$ = 7.52		**$Q^{m\ ave}$ = 2.30**
TENN-F-69053 NB	6–7 × (2.5–)3–3.5	6.75	2.00–2.33(–2.80)	2.16
TENN-F-48376 TN	(6–)6.5–9 × 2.5–3.5	7.40	2.00–3.20	2.59
TENN-F-47030 NY[2]	6.5–8.5 × 3–3.5	7.60	2.14–2.67	2.42
TENN-F-63806 VA[1]	6.5–9 × (3–)3.5–4.5	7.75	1.86–2.43(–3.00)	2.20
TENN-F-69073 NB	7–8.5 × 3.5–4	7.80	2.00–2.67	2.21
TENN-F-53522 NC	7–9.5 × 3–3.5	8.05	2.14–3.00	2.53
TENN-F-63806 VA[2]	7–9 × 4–4.5	8.05	1.67–2.25	1.95
WTU 021394 AK	7–8.5 × (2.5–)3–3.5	7.70	2.00–3.20	2.36
WTU 024005 AK	(6.5–)7–8.5 × (2.5–)3–3.5	7.85	2.17–2.85	2.58
WTU 021152 AK	(7–)8–9 × 3–4	8.30	2.13–2.67	2.42
		$L^{m\ ave}$ = 7.72		**$Q^{m\ ave}$ = 2.34**

[1] From pileipellis; [2] From hymenium; [3] Alaskan collections with European ITS sequences; [4] Herbarium designations according to Index Herbariorum (Thiers continuously updated)

often include small side lobes with narrow attachments to parent hyphae. It is easy to observe the erect hyphae which compose the spikes, but more difficult is observation of the subicular hyphae which produce the hyphal complexes which elongate into the spikes. The complexity and depth of the subiculum vary from arachnoid (and then revealing the color and glassy surface of the dried stipe cortex) (TENN-F-59282 SZ; TENN-F-59219 FR) to opaque-felty with subsumed spikes (TENN-F-67865 GE) to shaggy (TENN-F-67882 GE). The felty subiculum increases downward and appears as a sheath toward the stipe base, somewhat reminiscent of *Connopus acervatus*.

Pleurocystidia: Whether pleurocystidia are commonly present has not been thoroughly investigated. Structures resembling cheilocystidia were observed in three collections (TENN-F-59578 RU, TENN-F-67865 GE, TENN-F-59212 FR) among basidia rather than being clustered at the temini of lamellar tramal hyphae.

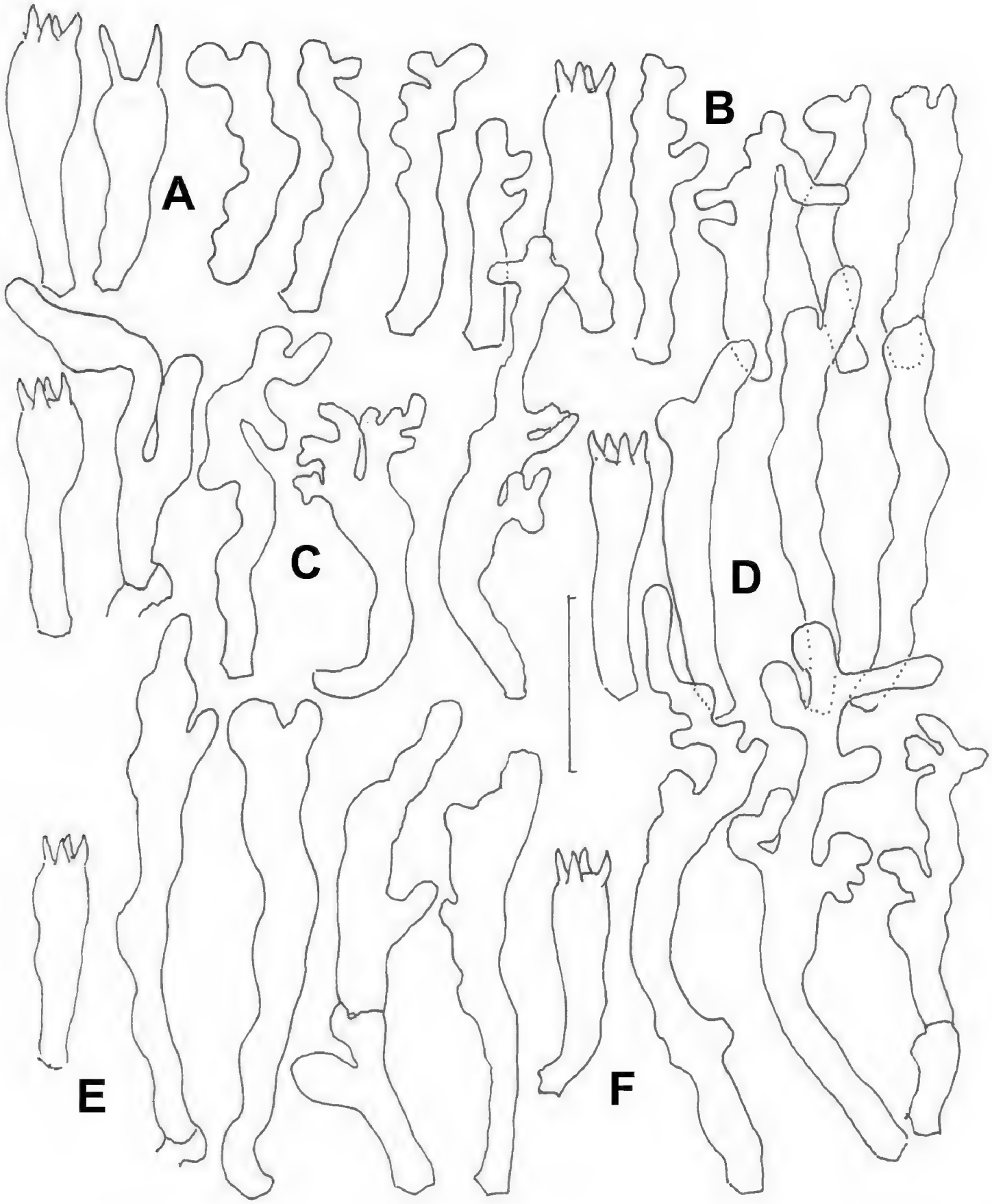

Figure 5. *Gymnopus confluens*. Europe. Outlines of cheilocystidia, showing variation in sizes and complexity. Basidia furnished for size comparison. **A** TENN-F-59285 SZ **B** TENN-F-59469 FI **C** TENN-F-53546 SW **D** TENN-F-59219 FR **E** TENN-F-67882 GE **F** TENN-F-60109 RU. Standard bar = 20 µm.

Basidia: Basidia varied little across both continents. Rarely, an individual two-spored basidium was detected, and four-spored basidia accounted for almost all mature basidia observed (Figs 5, 6).

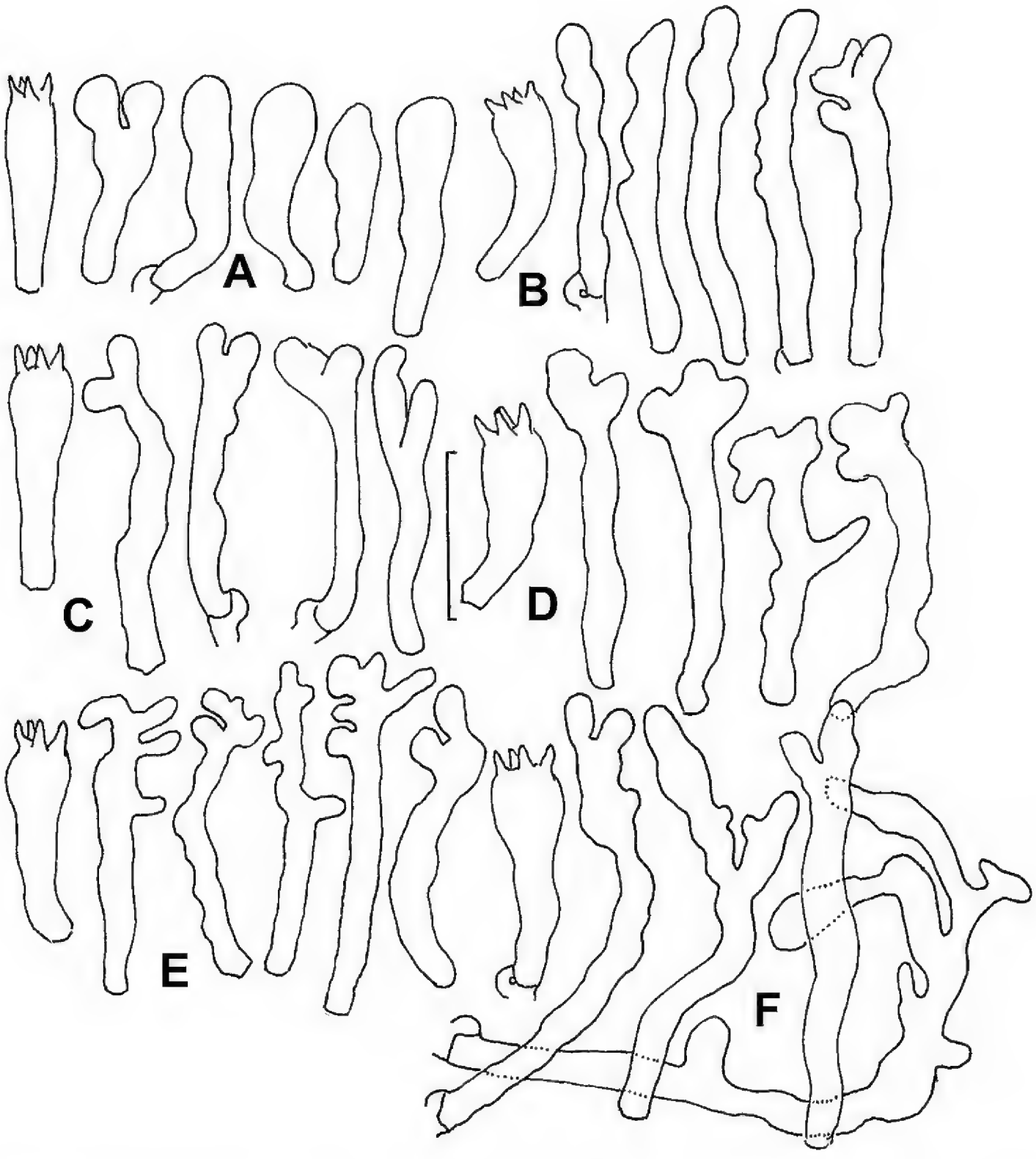

Figure 6. *Gymnopus confluens* subsp. *campanulatus* North America. Outlines of cheilocystidia, showing variation in sizes and complexity. Basidia furnished for size comparison. **A** MS4-009 NF **B** TENN-F-47030 NY **C** TENN-F-69053 NB **D** TENN-F-63806 VA **E** TENN-F-48376 TN **F** TENN-F-69074 NB.Standard bar = 20 µm.

A basidium is produced as a terminal cell of a subhymenial hypha. The hyphae then proliferates through the subtending clamp connection and another basidium is produced in the same fashion. After several such proliferations and basidial discharge (usually leaving little or no residue), the subhymenial hypha appears asymmetrically notched, and superficially resembles some cheilocystidia (TENN-F-67865 GE), for which they are easily mistaken, especially as seen in considerable numbers in older hymenia.

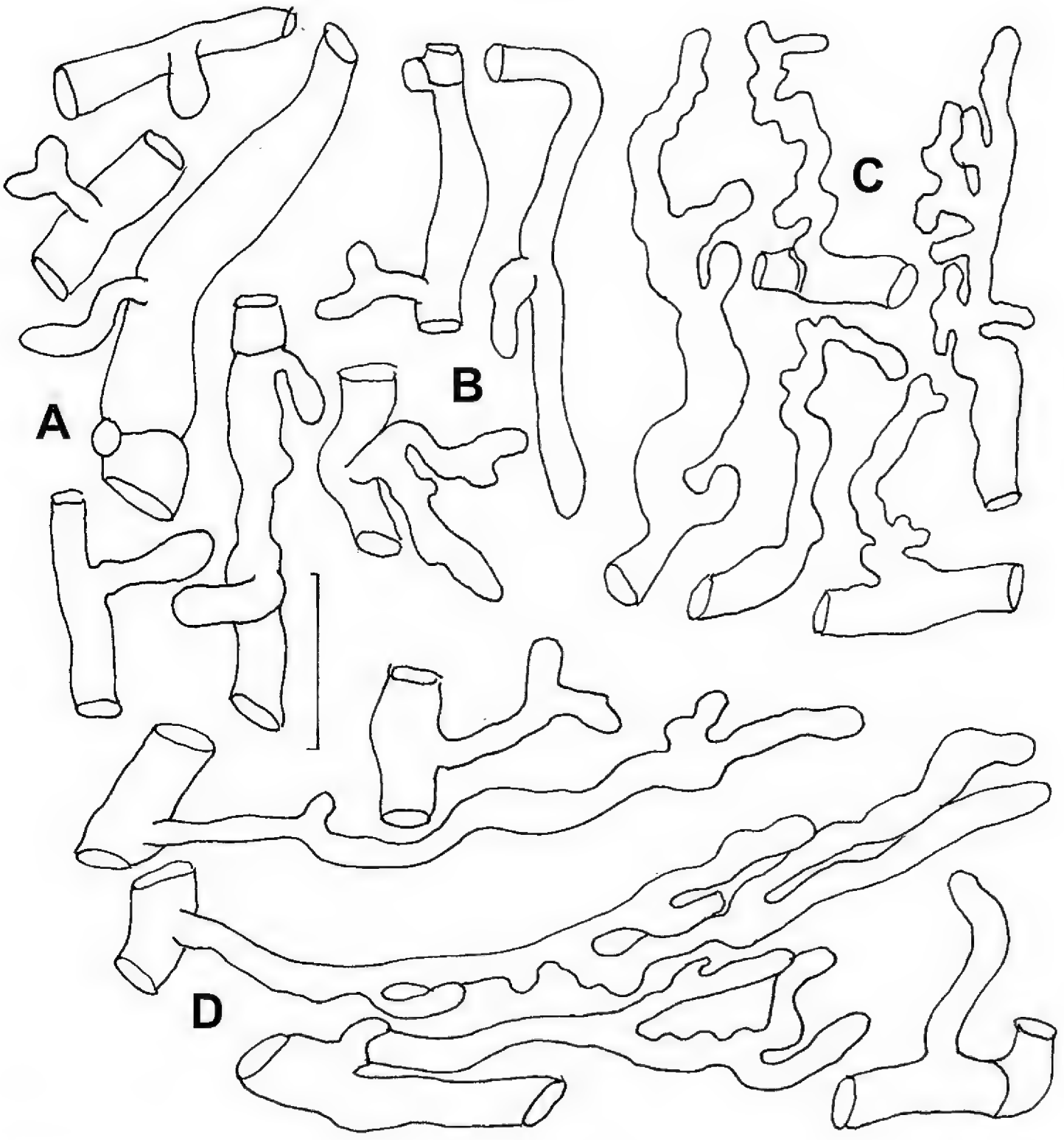

Figure 7. *Gymnopus confluens*. Europe. Outlines of side branches of pileipellis hyphae showing variation in size and complexity. **A** TENN-F-65131 BE **B** TENN-F-60109. RU **C** TENN-F-59469 FI **D** TENN-F-67865 GE. Standard bar = 20 µm.

Side branches from pileipellis: The side branches from pileipellis hyphae reported and illustrated by Halling (Halling 1983) and Antonín and Noordeloos (2010) were consistently observed. Shapes ranged from short, simple and digitate (i.e. TENN-F-13883, TENN-F-48376, TENN-F-69133, TENN-F-53522 from North America; TENN-F-65131, TENN-F-60109, TENN-F-67882, TENN-F-59469 from Europe) to over 100 µm long and branched in a tentacular fashion (i.e. TENN-F-63806 from North America; TENN-F-67865 from Europe). Quantitatively, however, these side branches seem longer (TENN-F-67882 GE, TENN-F-60109 RU) and usually more complex (i.e. branched in a coralloid fashion (TENN-F-67865 GE, TENN-F-59469

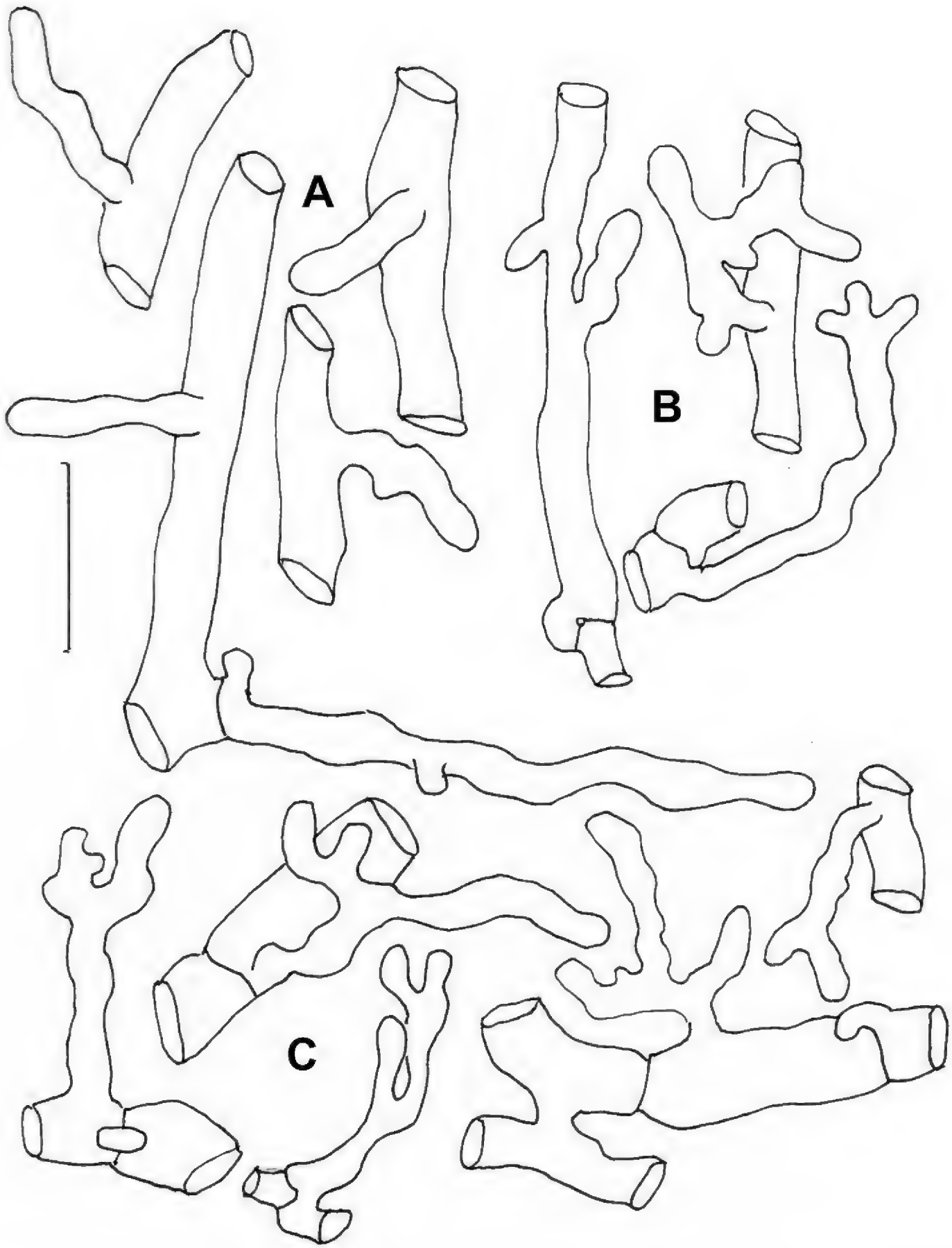

Figure 8. *Gymnopus confluens* subsp. *campanulate*. North American. Outlines of side branches of pileipellis hyphae, showing variation in size and complexity. **A** TENN-F-53522 NC **B** TENN-F-69133 NL **C** TENN-F-63806 VA. Standard bar = 20 µm.

FI) in European specimens than in American. It may be that this proliferation is aided by microscopic local moisture idiosyncratic to individual conditions (including some mucoid matrix if present). The variation was wide both within and between clades, and no clade separation was possible based on this character (Figs 7, 8).

Morphological summary: Morphological characteristics based on lamellae, side branches from pileipellis hyphae, cheilocystidia, caulocystidia, and pleurocystidia, varied considerably but observed differences were not continent-specific. Basidia and basidiospores showed little variability and did not differ between continents.

Sexual recognition experiments

Results of three self-crosses of *G. confluens* (collections TFB 7219, NC; 9048, CA; 11400, SZ) were reported (Mata et al. 2007) as showing tetrapolarity, not unusual in Omphalotaceae. This study added TENN-F-69053 (TFB14389), New Brunswick, Canada, which we also determined to be tetrapolar. In all cases, distribution of mating types was unbalanced and in most of these self-crosses, some SBIs were found which exhibited unexplained mating results, mostly ability to dikaryotize two opposing mating types, most easily explained by either harvesting of two hemicompatible basidiospore germlings together, or occurrence of two hemicompatible nuclei within a single basidiospore (Petersen 1995). Such results have also been found in other members of *Gymnopus* subg. *Vestipedes*, such as *G. subnudus* (Murphy 1992; Murphy and Miller 1993; Petersen 1995) which has been reported as bipolar and tetrapolar (Petersen, ined.)

In the sexual compatibility study using *G. confluens* [(Mata et al. 2007);Fig. 14], a total of 11 collections were used, seven from Europe and four from North America. Five intercontinental intercollection pairings were performed, all universally sexually compatible. If clamp connection production is accepted as a proxy for sexual reproduction, no apparent prezygotic reproductive barrier exists between the continents. It was concluded, based on limited evidence, that only a single sexual recognition species was involved. No attempt to segregate morphological entities was offered in that study.

Phylogenetic analyses

A PhyML tree based on ribosomal ITS sequences is given in Fig. 9. North American and European collections segregated largely into two distinct clades which share an average sequence identity of 96.75%. Collections from Alaska were placed in both clades. A collection from California was also affiliated with the European clade. For a smaller data set, both ribosomal ITS and LSU sequences were available and were concatenated. Results of the PhyML analysis are given in Fig. 10.

For the ITS data set, Rosenberg's P_{AB} statistic for both European and North American clades was $P=1.6 \times 10^{-8}$. Thus, a null hypothesis of reciprocal monophyly under a random coalescence model can be rejected. The probability of correctly identifying an unknown member of a putative species is given by P_{ID} statistics. P_{ID} (strict) European clade = 0.95 (σ=0.89,1.00) and P_{ID} (strict) North American clade = 0.98 (σ=0.93, 1.00). P_{ID} (strict) is the more stringent of the P_{ID} statistics. The probability P_{RD} that a clade has the observed distinctiveness under a null hypothesis of random coalescence for North

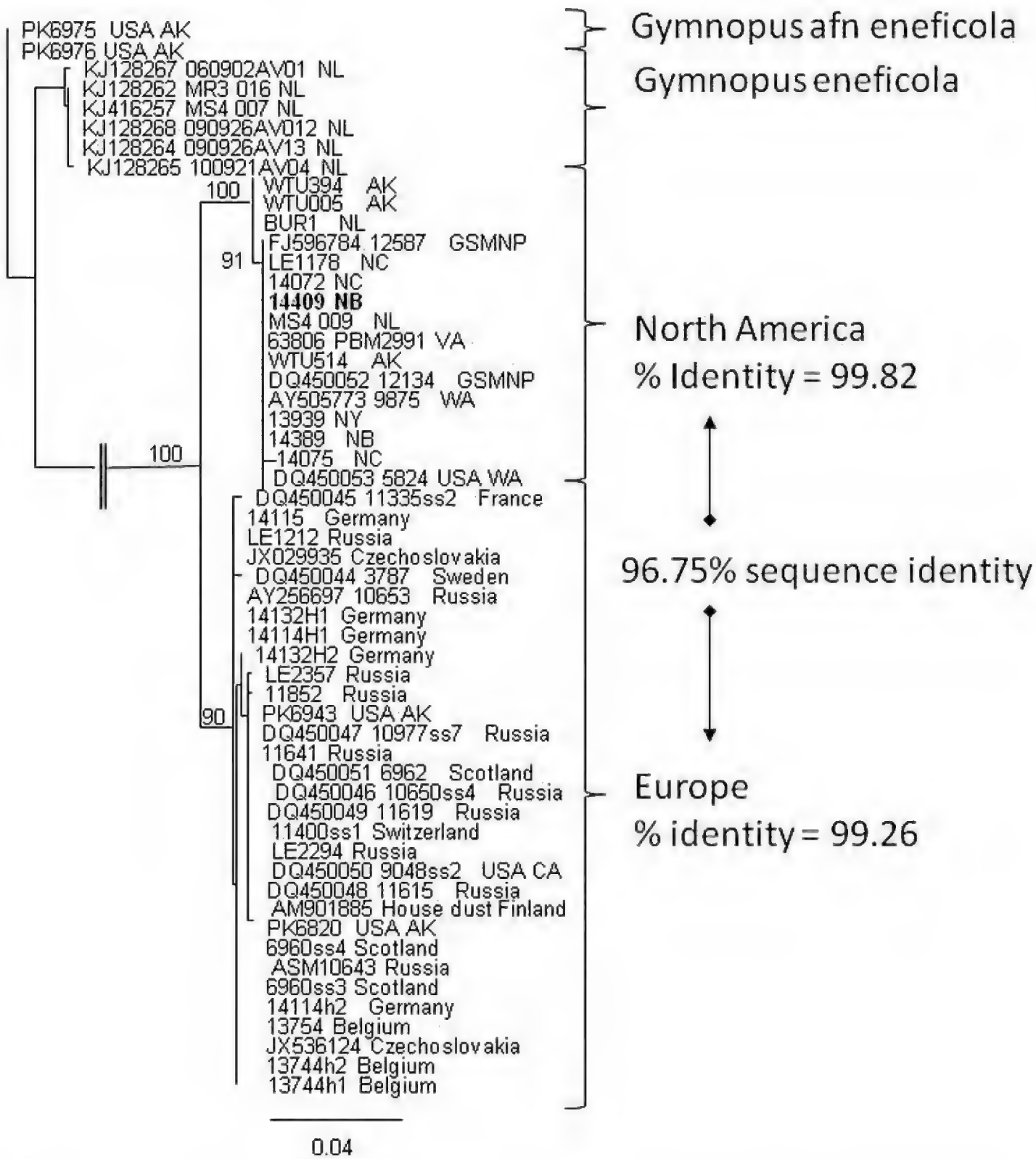

Figure 9. PhyML tree based on ribosomal ITS sequences. Bootstrap values based on 100 bootstrap replicates are at the left of the supported node. Analysis assumed the GTR model of evolution with the transition/transversion ratio, number of invariable sites and shape of the gamma distribution estimated. The log likelihood of the tree was -1776.7. Bold type = holotype of *Gymnopus confluens* subsp. *campanulatus*. Percent identity was based on the entire ITS1-5.8S-ITS2 sequence.

America is 0.05 and for Europe is 0.21. Neither of these probabilities reject the null hypothesis. PTP species-delimitation results produce both maximum likelihood and Bayesian estimates of the number of species. PTP for both analyses partitions *G. confluens* European and North American populations into two species groups but without significant support (bootstrap support for both analyses was 0.23 for North America and 0.55 for Europe. Outgroup taxa are also partitioned into two species groups.

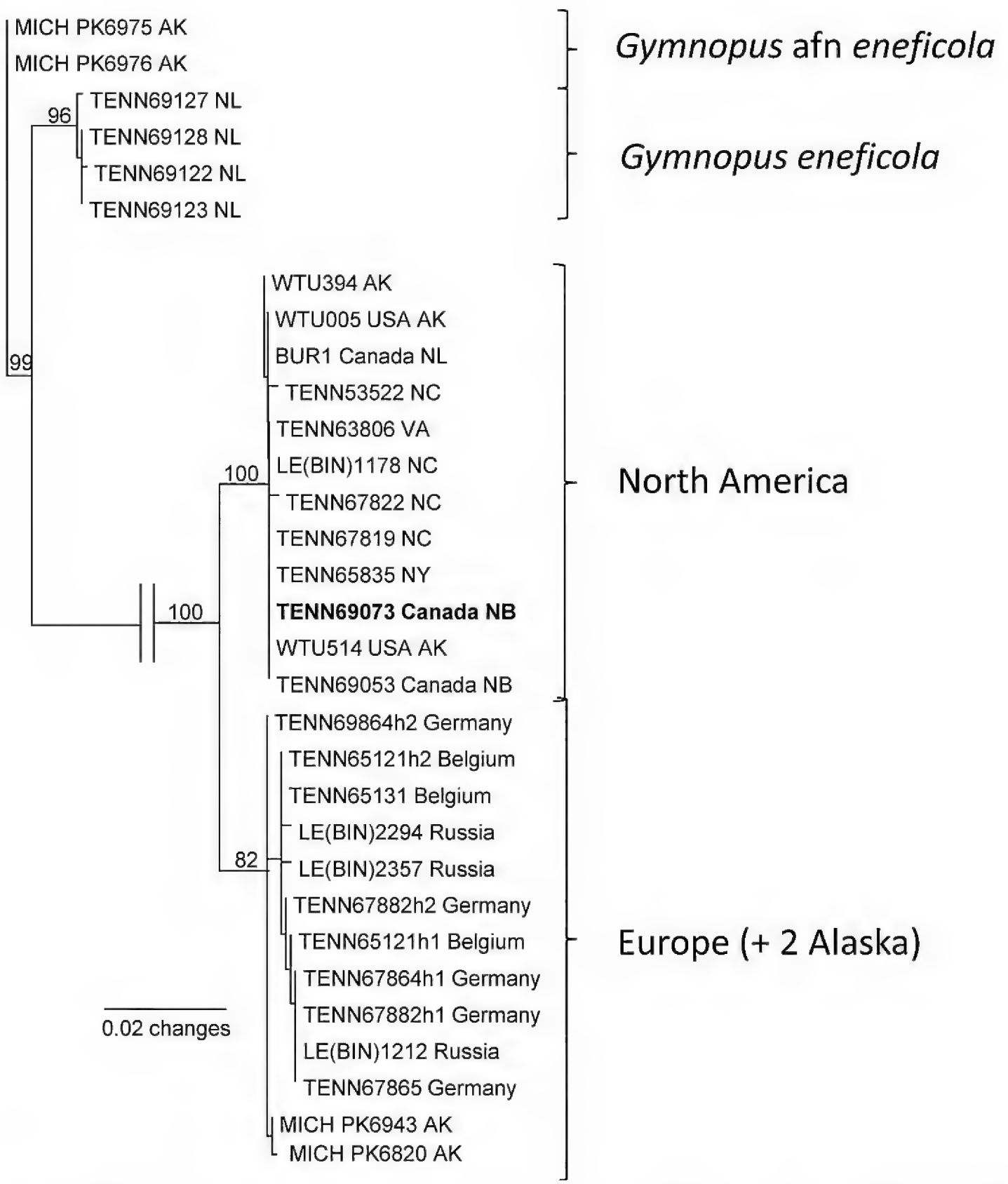

Figure 10. PhyML tree based on concatenated ribosomal ITS + LSU sequences. Bootstrap values based on 100 bootstrap replicates are at the left of the supported node. Analysis assumed the GTR model of evolution with the transition/transversion ratio, number of invariable sites and shape of the gamma distribution estimated. The log likelihood of the tree was -3135.8. Bold type = holotype of *Gymnopus confluens* subsp. *campanulatus*.

Discussion

Allopatric speciation may be the most common mode of speciation in fungi and other organisms, and separation of populations on different continents would, in the absence of significant gene flow, lead gradually to accumulation of genetic differences

and ultimately speciation. The mode and tempo of speciation, however, must vary with reproductive strategies and selection pressures. The point at which two allopatric populations become new species is often a matter of judgment but important in terms of evaluating conservation status and estimating species diversity for a given region. Numerous studies suggest that in basidiomycete fungi, ability of allopatric populations to intercross *in vitro* is conserved as a function of the unique multiple allelic mating systems even while genetic divergence (usually indicated by differences in nuclear ribosomal ITS sequences) proceeds (Gordon and Petersen 1997; 1998; Grubisha et al. 2012; James et al. 1999; Lickey et al. 2002; Lickey et al. 1999; Taylor et al. 2006; Vilgalys and Sun 1994). It should be noted, however, that *in vitro* compatibility examines only one aspect of reproductive intercompatibility and that failure to produce F_1 fruitbodies, reduced fertility of F_1 hybrids, lack of or inviability of F_2 progeny and competition failure at any level may also be involved in reproductive isolation. These factors have not been evaluated as isolated mechanisms between basidiomycete populations to date.

In *Gymnopus confluens*, two of three criteria used to evaluate delineation of species (morphology and ability to intercross *in vitro*) show no significant intercontinental separation. ITS sequences, however, are divergent (3.25% base pair difference), a level often used to suggest different species (Hughes et al. 2009), and European and North American clades are well-supported. The combination of a barcode gap, Rosenberg's P_{AB} statistic results and P_{ID} (strict) suggest that North American and European clades are monophyletic, that the observed differences are not due to coalescence in gene trees and that they are distinct phylogenetic species. In contrast, P_{RD} and PVP do not reject the possibility that the observed result (Fig. 9) is due to random coalescence. The question then becomes whether to assign these populations nomenclatural rank on the basis of ribosomal ITS and LSU sequences alone. To identify these populations as comprising a single species ignores significant ITS + LSU sequence divergence indicative of speciation processes, and underestimates diversity. To identify these populations as separate species may overstate the degree of genetic divergence. A middle-ground solution seems to be to assign these populations subspecies rank, thus recognizing genetic differentiation. We do so below adhering to, insofar as possible, procedures suggested by Tripp and Lendemer (2014) for naming taxa when molecular evidence is the only evidence available.

The finding that ITS sequences for collections from Alaska represented two distinct ITS entities, one of which falls within the European clade (Fig. 9) suggests a dual origin for collections from this region. An ITS sequence of a collection from California also falls within the European clade. Possibly, these collections represent human-mediated transfer of material from Europe to North America. Alternately, movement from Eurasia to Alaska thence to California may have been feasible via the Bering land bridge during periods of glaciation, but without an understanding of *G. confluens* from Asia, neither hypothesis be substantiated. Intra-continental geographical partitioning is not clearly evident for either European or North American populations of *Gymnopus confluens*. This contrasts with findings in some other basidiomycete taxa (Geml et al. 2008; Hughes et al. 2014; Zhao et al. 2013) but intracontinental biogeographical distributions have not been extensively examined and are likely to be species-specific.

Taxonomy

***Gymnopus confluens* subsp. *campanulatus* (Peck) R.H. Petersen, comb. et stat. nov.**
MycoBank no. 811950

Basionym: *Collybia confluens* var. *campanulatus* Peck. "1901" (1902). Bull. N.Y. State Mus. 54: 963.

Type material. Holotype. United States, New York, Bolton, IX.1900, coll. C.H. Peck (NYS). **Epitype.** CANADA, New Brunswick, Fundy Nat. Park, vic. Alma, Caribou Plains Trail, 45°38.587' N, 65°06.937' W, 25.IX.2013, coll Stephen Clayden, TFB14409 (TENN-F-69073)

Taxon diagnosis. 1) ITS nrDNA sequence significantly different from sequence of *Gymnopus confluens* subsp. *confluens*; 2) basidiomata densely gregarious to subcespitose; 3) basidiomata apparently persistent beyond spore production and discharge; 4) stipe:pileus diameter ration from 2-5:1 (stipe significantly longer than pileus diameter); 5) pileus hygrophanous, brown where moist, pallid tan to pinkish buff where dry, drying to more uniform pallid color; 6) lamellae very crowded (total lamellae at pileus margin 110-140), shallow, seceding upon drying; 7) lamellar edge entire (smooth) to delicately fimbriate; 8) stipe grooved or compressed, stiff, with brown cortex (rind); 9) stipe vesture concolorous with pileus when moist and fresh, easily bleaching on drying to pallid gray shades; 10) basidiospores generally elongate-ellipsoid to sublacrymiform; 11) cheilocystidia stalked, usually lobed or strangulate, sometimes branched; 12) pileipellis hyphae smooth, firm-walled, with occasional to common side branches appearing digitate to long and branched. 13) Distribution in North America.

Description. Gymnopus confluens subsp. campanulatus; taxon description:

Pileus: Pileus 7–33 mm broad, thin (parchment-like and brittle when dry), often generally truncate-conical to shallowly convex with downturned margin when young becoming applanate to somewhat flaccid campanulate by maturity, occasionally with very shallow umbo or flattened over disc, minutely suede-like (not glabrous); disc "cinnamon buff" (6B4; dry), "sayal brown" (6C5) to "tawny olive" (5C5; moist); limb and margin "pinkish buff" (6A3) to "tilleul buff" (7B2) occasionally in hygrophanous zones; margin entire to somewhat lobate, sometimes subtly closely striate when dry. **Lamellae:** Lamellae very crowded, free to adnexed but significantly seceding upon drying and leaving a pale, off-white ring around the stipe apex, with relatively numerous lamellulae, very shallow (1 mm or less deep), slightly thickish, "tilleul buff" (7B2), "light buff" (3A2) to "deep olive buff" (3C3); lamellar edge never totally smooth, minutely fimbriate to minutely serrulate and usually paler than lamellar face. **Stipe:** Stipe of mature basidiomata 35–80(-95) mm long, 2.5–4 mm broad, stiff, equal except for slightly expanded base and slightly flaring apex, consistently grooved or fluted (but not compressed), stuffed to profoundly hollow; cortex (rind) tough, russet to mahogany ("Mars brown" 8F7, "tawny olive" 5C5), glassy; medulla (interior), lightly stuffed, nearly hollow, grayish cream colored, loose; vesture more or less uniform over

entire stipe length, consistently "tilleul buff" (7B2) to "pale olive buff" (3B2) when dry, detersile when fresh, easily disarticulated by handling when dry into minute chaff. Vesture of luxuriant form (New Brunswick, TFB 14409) delicately pruinose, apically concolorous with gills, soon "sayal brown"(6C5) to "Verona brown" (6E5). **Odor** none to faintly fresh; **taste** negligible to mild, perhaps weakly acidic, NOT acrid.

Habitat and phenology. on duff under *Quercus* and other hardwoods including *Acer* (TENN 63806); gregarious on leaf litter under *Fagus* (TENN 47030) and occasionally *Pinus*; hardwood duff (TENN 48376).

Pileipellis a thin layer of generally radially oriented hyphae; hyphae 4–11 µm diam, firm-walled, smooth (unornamented) to hardly ornamented (minute grit with suggestion of stripes or rings), conspicuously clamped, with infrequent, erect, side branches, ->75 µm long, 1.5–2.5 µm diam, simple to branched similar to cheilocystidial apices, arising from clamp connection or between clamps, often terminating in gradually tapering (2–4 µm diam at terminus) hyphal tips; contents heterogeneous, from amorphous sludge to coarsely spotted (PhC). **Pileus and lamellar trama hyphae** 3-9 µm diam, thin- to firm-walled, with occasional cheilocystidioid branches which seem to arise from clamp connections, conspicuously clamped, essentially free-form (TENN 53522), often anastomosing in "H" connections, when squashed often liberating minute debris in a subsoluble mucoid substance. Basidioles 22–25 × 5-7 µm, narrowly fusoid to torpedo-shaped, arising from a clamp. **Basidia** 21–30 ×7–9(-10) µm, clavate to broadly clavate, seldom bulbo-clavate, obscurely clamped, 4-sterigmate, arising from an obscure clamp; contents more or less homogeneous. **Basidiospores** (6-)6.5–9 × (2.5-)3–3.5(-4) µm (Q = 2.00-3.20; Q^m = 2.59; L^m = 7.40 µm), elongate ellipsoid, somewhat flattened adaxially to slightly sway-back, thin-walled, smooth; contents homogeneous. In TFB 14409 (NB), spores plump ellipsoid to plump pip-shaped; contents 1-several guttulate. TFB 14389 (NB) produced somewhat smaller basidiospores [6–7 × (2.5-)3–3.5 µm (Q = 2.00-2.33(-2.80); Q^m 2.16]. Lamellar edge entire to minutely fimbriate or minutely serrulate with cheilocystidia (64X), under magnification, lamellar edge fertile, with cheilocystidia locally abundant to sparsely scattered amongst fertile basidia; **cheilocystidia** typically (23-)34–77 × 2.5–4(-15) µm, hyphal, often 2-celled (with internal clamp), simple and substrangulate to usually branched with apical or subapical lobes or coralloid, contorted branches. Usually an accumulation of subsoluble mucoid material (with granular inclusions and embedded spores) surrounding cheilocystidial apices, perhaps exuded by the cheilocystidia themselves; cheilocystidia occasionally ramifying into slender (~1.5 µm diam), branched, arbuscular hyphal tips seemingly embedded in the mucoid matrix. Stipe surface hyphae 3.5–9 µm diam, strictly longitudinal and tightly parallel, occasionally but conspicuously clamped, often irregularly beset with small side lobes and short branchlets, sometimes arising from a clamp with a very thin mucoid sheath (with abundant embedded granular or globular material). **Stipe vesture** juxtaposed to stipe surface a thick, tightly interwoven thatch of thick-walled (wall -0.7 µm thick), very frequently branched, abundantly clamped hyphae 3.5–4.5 µm diam from which vesture columns and/or spikes arise; columns or spikes -100 µm tall, do not appear coherent, nor do

they seem gathered from neighboring hyphae, but seem to arise in groups to form columns; **caulocystidial hyphae** -150 × 3.5–5 µm, thick-walled (wall -0.7 µm thick) at origin, soon branched (at a clamp) to produce two individuals, often with an additional internal clamp and further unbranched, firm-walled, conspicuously clamped, replete with numerous small lobes or branches, terminating in a bluntly rounded apex.

Conclusions

Gymnopus confluens in Europe and North America shows intercontinental but not intracontinental divergence in ITS and LSU sequences but European and North American populations do not differ morphologically and retain the ability to dikaryotize *in vitro*. Intercontinental ITS/LSU sequence divergence is sufficient to recognize differences taxonomically. The North American population is described as *G. confluens* subsp. *campanulatus*.

Acknowledgements

We thank Dr. Nadezhda Psurtseva (Komarov Botanical Institute of the Russian Academy of Sciences) for arranging field work in Russia and for cultures from LE-BIN. We thank Stephen Claydon for donating specimens found during the Fundy Foray (2013) and Dr. Felix Hampe for collections found at the Russulales Congress in Germany 2012. MICH and WTU loaned critical specimens for analysis. Mr. Matt Aldrovandi helped with sequencing. We thank Roy Halling, Karl-Henrik Larsson, Christian Wurzbacher and an unknown reviewer for their reviews and suggestions.

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